Differential Regulation of ZEB1 and EMT by MAPK-Interacting Protein Kinases (MNK) and eIF4E in Pancreatic Cancer

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Abstract

Human pancreatic ductal adenocarcinoma (PDAC) tumors are associated with dysregulation of mRNA translation. In this report, it is demonstrated that PDAC cells grown in collagen exhibit increased activation of the MAPK-interacting protein kinases (MNK) that mediate eIF4E phosphorylation. Pharmacologic and genetic targeting of MNKs reverse epithelial–mesenchymal transition (EMT), decrease cell migration, and reduce protein expression of the EMT-regulator ZEB1 without affecting ZEB1 mRNA levels. Paradoxically, targeting eIF4E, the best-characterized effector of MNKs, increases ZEB1 mRNA expression through repression of ZEB1-targeting miRNAs, miR-200c and miR-141. In contrast, targeting the MNK effector hnRNPA1, which can function as a translational repressor, increases ZEB1 protein without increasing ZEB1 mRNA levels. Importantly, treatment with MNK inhibitors blocks growth of chemoresistant PDAC cells in collagen and decreases the number of aldehyde dehydrogenase positive (Aldefluor⁺) cells. Significantly, MNK inhibitors increase E-cadherin mRNA levels and decrease vimentin mRNA levels in human PDAC organoids without affecting ZEB1 mRNA levels. Importantly, MNK inhibitors also decrease growth of human PDAC organoids.

Implications: These results demonstrate differential regulation of ZEB1 and EMT by MNKs and eIF4E, and identify MNKs as potential targets in pancreatic cancer. Mol Cancer Res; 14(2); 216–27. © 2015 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth leading cause of cancer-related deaths in the United States, with a median survival of approximately 6 months and 1-year survival of approximately 20% (1, 2). The continuing dismal outcome is attributed to the fact that PDAC is an aggressive cancer. Contributing to the aggressive nature of this cancer is the intense fibrotic reaction that is associated with the primary tumor and the metastatic PDAC lesions (3, 4). The fibrotic reaction, which can account for over 80% of the tumor mass (4, 5), contains extensive amounts of fibrillar collagen. We have previously shown that PDAC cells respond to type I fibrillar collagen by increasing motility, decreasing epithelial markers, and upregulating mesenchymal markers (6–8).

PDAC tumors are also associated with dysregulation of mRNA translation of proinvasive genes that contributes to tumor progression (9, 10). In addition, increased phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) in human PDAC tumors is associated with high-grade tumors and poor prognosis (9). The function of eIF4E is critical for malignant transformation and promotes tumor development in vivo (11, 12). The MAPK-interacting protein kinases 1 and 2 (MNK1 and MNK2) mediate eIF4E phosphorylation on Ser209 and regulate eIF4E-mediated mRNA translation (13, 14). An additional MNK effector is hnRNPA1 (heterogeneous nuclear ribonucleoprotein A1), which has previously been shown to function as a translational repressor of some genes (15, 16). Because of the importance of mRNA translation in tumorigenesis (17, 18), a better understanding of the contribution of MNKs to pancreatic cancer progression may provide a targeted approach for the treatment of PDAC patients.

In this report, we show that PDAC cells grown in three-dimensional (3D) type I collagen demonstrate increased eIF4E phosphorylation. PDAC cells that have undergone epithelial–mesenchymal transition (EMT) also demonstrate increased activation of MNKs in collagen. Pharmacologic and genetic targeting of MNKs reverses EMT, decreases cell migration, and reduces protein expression of the EMT-regulator ZEB1 without affecting ZEB1 mRNA levels. Significantly, MNK inhibitors increase E-cadherin mRNA levels and decrease vimentin mRNA levels in human pancreatic organoids without affecting ZEB1 mRNA levels. Paradoxically, targeting eIF4E increases ZEB1 mRNA and protein expression. In contrast, targeting the MNK effector hnRNPA1...
increases ZEB1 protein without increasing ZEB1 mRNA levels. Importantly, treatment with MNK inhibitors blocks growth of chemoresistant PDAC cells in collagen, inhibits growth of PDAC organoids, and decreases the number of Aldehydeur (+) cells, suggesting that MNKs may regulate cancer stem cells and may be potential targets in pancreatic cancer.

Materials and Methods

Reagents

General tissue culture materials were obtained from VWR International. Antibodies against eIF4E, tubulin, HSP90, ZEB1, and Dicer were obtained from Santa Cruz Biotechnology, whereas antibodies against p-eIF4E, MNK1, p-MNK1, and Drosha were purchased from Cell Signaling Technology. Anti-GAPDH antibody was from Abcam. Secondary antibodies were purchased from Cell Signaling Technology. siRNAs against MNK1 and MNK2 were purchased from Dharmacon, ZEB1 siRNA was obtained from Life Technologies, whereas eIF4E and hnRNPA1 siRNAs were from Santa Cruz Biotechnology. The Aldefluor Assay Kit was purchased from Stemcell Technologies.

Cell culture

AsPC1, CD18/HPAF-II, and Panc1 cells were obtained from the ATCC. Cells were maintained in DMEM containing 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Chemoresistant CD18 (CD18-CR) cells were generated by treating parental CD18 cells with increasing concentration of 5-fluorouracil (5-FU) over a period of 3 months (19). The surviving cells were maintained in 10 μmol/L concentration of 5-FU. The CD18 and CD18-CR cells were authenticated by short tandem repeat profiling at the Johns Hopkins Genetic Resources Core Facility in October 2013, whereas AsPC1 and Panc1 cells were authenticated in June 2010.

Embedding and examination of cells in 3D type I collagen gels

Collagen mixture (2 mg/mL) was made by adding the appropriate volumes of sterile water, 10X DMEM, and NaOH and kept on ice until needed (8, 20). Cells were then suspended in the collagen solution and allowed to gel at 37°C. For protein analysis, the collagen gels were treated with collagenase to extract cells for Western blotting. For morphologic examination of cells, cell colonies in 3D collagen were examined using a Zeiss Axiovert 40 CFI. microscope and pictures taken with a Nikon Coolpix 4500 camera (8). The relative size of individual colonies was measured using ImageJ.

Transfection

Cells were transfected with siRNA against MNK1, MNK2, ZEB1, eIF4E, or control siRNA using RNAiMax (Invitrogen) according to the manufacturer’s instructions before plating into collagen (8).

Quantitative real-time PCR analysis

Quantitative gene expression was performed with gene-specific probes as described previously (8, 20). Similarly, expression of mir-200a/b/c, mir-141, and RNU48 was analyzed as previously published (21).

Isolation of polysomal RNA

Polyosomal fractionation was performed as previously described (22, 23). Briefly, cell pellets were lysed in hypotonic polysomal lysis buffer, clarified by centrifugation, and optical density (OD) at 260 nm was measured for each of the supernatant samples. DMSO- and CGP57380-treated supernatants containing 300 OD were then layered over 10% and 50% continuous sucrose gradients. Following ultracentrifugation, the fractions were collected while monitoring the absorbance at 254/260 nm as a function of gradient depth. The polyosomal fractions were pooled, total RNA from polyosomal fractions was isolated, and the levels of ZEB1 and GAPDH mRNA in the polyosomal fractions and in the whole-cell lysates were determined by qRT-PCR. The relative amounts of ZEB1 mRNA in the polyosomal fractions were then compared with the relative amounts of ZEB1 mRNA in the whole-cell lysates.

Human PDAC tissue analysis

Pancreatic tissue was obtained from patients with pancreatic adenocarcinoma on an Institutional review board (IRB)-approved protocol. The tissue microarray specimens were stained with p-eIF4E antibody (Abcam), and also trichrome stained to assess for fibrosis (6).

Human PDAC organoids

Deidentified human PDAC tumor specimens were processed using the recently published Tuveson Laboratory protocol (24). Briefly, the tumors were minced and digested with collagenase II and TrypLE, embedded in growth factor–reduced Matrigel, and maintained in human complete media (24). To examine the effects of targeting MNKs on gene expression in these organoids, organoids were treated with DMSO or CGP57380 for 36 hours, and the effect on gene expression was determined by qRT-PCR.

Immunoblotting

Immunoblotting for p-eIF4E, eIF4E, p-MNK1, MNK1, E-cadherin, vimentin, ZEB1, Dicer, Drosha, hnRNPA1, GAPDH, HSP90, and tubulin was done as previously described (8, 20).

Statistical analysis

All statistical analyses were done using Microsoft Excel or GraphPad Instat using a two-tailed t test analysis. Error bars represent SEs.

Results

Collagen-dependent phosphorylation of eIF4E in PDAC cells

Increased eIF4E phosphorylation on Ser209 in human PDAC tumors correlates with higher-grade tumors and worse prognosis (9). Because increased fibrosis can be associated with worse prognosis in human PDAC tumors (3, 4), we examined the effect of the collagen microenvironment on eIF4E phosphorylation. As shown in Fig. 1A, PDAC cells in 3D collagen demonstrate increased eIF4E phosphorylation on Ser209. Because MNKs are known to phosphorylate eIF4E on Ser209 in other systems (25, 26), we examined the effects of the MNK inhibitor CGP57380 on collagen-induced eIF4E phosphorylation. Initially, we examined the effect of the collagen microenvironment on MNK phosphorylation/activation. PDAC cells in collagen demonstrated increased MNK1 phosphorylation on Thr197/202...
Collagen-dependent phosphorylation of eIF4E in PDAC cells. A, PDAC cells (AsPC1, CD18, and Panc1) were grown on tissue culture plastic or in 3D type I collagen (2 mg/mL) for 24 hours. The cell lysates were analyzed for eIF4E phosphorylation on Ser209 (p-eIF4E) by Western blotting. B, PDAC cells were grown for 24 hours on tissue culture plastic or in 3D type I collagen (2 mg/mL). The cell lysates were analyzed for eIF4E phosphorylation on Thr197/202 by Western blotting. C, PDAC cells growing in 3D type I collagen were treated with DMSO or CGP57380 (2.5 μmol/L) for 24 hours and the effect on eIF4E phosphorylation was determined by Western blotting. D, PDAC cells were transfected with control siRNA (siCtrl) or a combination of MNK1- and MNK2-specific siRNAs (siMNK1/2) for 48 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The effect on MNK1 and MNK2 mRNA expression was determined by qRT-PCR. E, human pancreatic TMA samples containing 27 pancreatic tumor specimens were immunostained with anti-p-eIF4E antibody and assessed for fibrosis using trichrome staining. The relative immunostaining and the extent of fibrosis were graded as low (0 or 1+) or high (2+ or 3+). The relationship between p-eIF4E expression in the tissue samples and the extent of fibrosis was assessed by the Fisher exact test.

Figure 1.

Collagen-dependent phosphorylation of eIF4E in PDAC cells. A, PDAC cells (AsPC1, CD18, and Panc1) were grown on tissue culture plastic or in 3D type I collagen (2 mg/mL) for 24 hours. The cell lysates were analyzed for eIF4E phosphorylation on Ser209 (p-eIF4E) by Western blotting. B, PDAC cells were grown for 24 hours on tissue culture plastic or in 3D type I collagen (2 mg/mL). The cell lysates were analyzed for eIF4E phosphorylation on Thr197/202 by Western blotting. C, PDAC cells growing in 3D type I collagen were treated with DMSO or CGP57380 (2.5 μmol/L) for 24 hours and the effect on eIF4E phosphorylation was determined by Western blotting. D, PDAC cells were transfected with control siRNA (siCtrl) or a combination of MNK1- and MNK2-specific siRNAs (siMNK1/2) for 48 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The effect on MNK1 and MNK2 mRNA expression was determined by qRT-PCR. Effect on MNK1 protein levels and eIF4E phosphorylation was determined by Western blotting. D, PDAC cells were transfected with control siRNA (siCtrl) or a combination of MNK1- and MNK2-specific siRNAs (siMNK1/2) for 48 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The effect on MNK1 and MNK2 mRNA expression was determined by qRT-PCR. Effect on MNK1 protein levels and eIF4E phosphorylation was determined by Western blotting.

Collagen-dependent phosphorylation of eIF4E in chemoresistant PDAC cells

It is now well recognized that cells that have undergone EMT are more invasive and metastatic (27, 28). Because cells developing resistance to chemotherapy can undergo EMT (27), we treated CD18 cells with increasing concentrations of 5-FU chemotherapy to generate chemoresistant (CD18-CR) cells. These cells demonstrated loss of E-cadherin and increased expression of vimentin (Fig. 2A), indicating that the CD18-CR cells have undergone EMT. CD18-CR cells also showed increased expression of ZEB1 (Fig. 2A), a well-known regulator of EMT (27, 29, 30). The CD18-CR cells were also more migratory on the collagen-coated surfaces (Fig. 2B). Although CD18-CR cells demonstrated reduced levels of eIF4E phosphorylation compared with CD18 cells (Fig. 2C), the CD18-CR cells also demonstrated increased eIF4E and MNK phosphorylation when grown in 3D collagen (Fig. 2C and Supplementary Fig. S1). Furthermore, treatment with the MNK inhibitor CGP57380, or transfection with MNK1/2 siRNAs, blocked collagen-induced eIF4E phosphorylation in CD18-CR cells (Fig. 2D and E).

Targeting MNKs increases E-cadherin, decreases vimentin, and reduces migration of PDAC cells

We next examined the effect of pharmacologic targeting of MNKs on E-cadherin and vimentin protein and mRNA levels in CD18-CR cells. Treatment with the MNK inhibitor CGP57380 increased E-cadherin protein and mRNA levels and decreased vimentin protein and mRNA levels (Fig. 3A). Treatment with CGP57380 also decreased migration of CD18-CR cells on collagen-coated surfaces (Fig. 3B). We also evaluated the relative role of MNK1 and MNK2 in regulating EMT by downregulating MNK1, MNK2, or both MNK1 and MNK2. Downregulating either MNK1 or MNK2 decreased eIF4E phosphorylation (Fig. 3C); however, combined downregulation of MNK1 and MNK2 completely blocked eIF4E phosphorylation (Fig. 3C). Combined knockdown of MNK1 and MNK2 particularly increased E-cadherin mRNA and protein levels (Fig. 3C and D). Although MNK1 knockdown had minimal effect on vimentin mRNA and protein levels, MNK2 knockdown decreased vimentin protein and mRNA levels (Fig. 3C and D). Moreover, the effect of MNK2 siRNA on vimentin levels was not further affected by cotransfection with MNK1 siRNA. These results suggest that MNK2 in particular regulates EMT in PDAC cells.
Targeting MNKs decreases the protein expression of ZEB1 without reducing ZEB1 mRNA levels

We next evaluated the effect of targeting MNKs on ZEB1 protein and mRNA levels. Initially, we examined the effect of ZEB1 siRNA in reversing EMT and attenuating migration. Downregulating ZEB1 restored E-cadherin expression in CD18-CR cells (Fig. 4A), and reduced migration of CD18-CR cells on collagen-coated surfaces (Fig. 4B). Significantly, treatment with the MNK inhibitor CGP57380 decreased ZEB1 protein levels without affecting ZEB1 mRNA levels in CD18-CR and AsPC1 cells (Fig. 4C). Moreover, knockdown of MNK1, MNK2, or both MNK1 and MNK2 did not affect ZEB1 mRNA levels (Fig. 4D). However, while MNK1 siRNA had minimal effect on ZEB1 protein levels, MNK2 siRNA decreased ZEB1 protein levels (Fig. 4D). These results indicate that MNK2 in particular regulates EMT by blocking translation of ZEB1 mRNA in pancreatic cancer cells.

We also examined the effect of CGP57380 on ZEB1 mRNA translation using polysomal analysis. CD18-CR cells were treated with CGP57380 for 48 hours, polysomal mRNA was isolated, and the effect on ZEB1 mRNA in the polysomal fractions was determined. As shown in Fig. 4E, ZEB1 mRNA
levels in the polysomal fractions were decreased following treatment with CGP57380, thus further suggesting that MNKs regulate ZEB1 mRNA translation in pancreatic cancer cells.

**Targeting eIF4E increases ZEB1 protein and mRNA levels and decreases ZEB1-targeting miR-200c and miR-141 miRNAs**

Because eIF4E is the best-characterized target of MNKs (13, 14), we examined the effect of downregulating eIF4E on EMT in CD18-CR cells and AsPC1 cells. Paradoxically, downregulation of eIF4E increased ZEB1 mRNA and protein levels in CD18-CR cells (Fig. 5A), and in AsPC1 and Panc1 cells (Supplementary Fig. S2). The increase in ZEB1 levels was associated with a decrease in E-cadherin mRNA levels and an increase in vimentin mRNA and protein levels (Fig. 5A). Consistent with increased ZEB1 levels, eIF4E knockdown cells demonstrated a more "fibroblastic" phenotype (Fig. 5A). In contrast, MNK1/2 knockdown cells, which have decreased ZEB1 (Fig. 4), showed a more rounded phenotype (Fig. 5A).

It is now well established that the miR-200 family of miRNAs can regulate ZEB1 expression (30, 31). The miR-200 family is composed of two clusters of miRNA located on two different chromosomes, one on chromosome 1 at 1p36 (miR-200b, miR-200a, and miR-429) and the other on chromosome 12 at 12p13 (miR-200c and miR-141; refs. 32, 33). Thus, to understand the differential effect of eIF4E and MNK1/2 on ZEB1 expression, we evaluated the effect on miR-200b and miR-200a on chromosome 1, and on miR-200c and miR-141 on chromosome 12. Although eIF4E knockdown in pancreatic cancer cells did not
affect the levels of pri-miRNA transcripts of miR-200b, miR-200a, or miR-200c-141 (Supplementary Fig. S3), there was 70% and 80% decrease in miR-200c and miR-141 levels (Fig. 5B). To demonstrate that these miRNAs regulate ZEB1 in CD18-CR cells, we transfected the cells with pre-miR-200c and pre-miR-141 and evaluated the effect on ZEB1, E-cadherin, and vimentin. Increasing the levels of miR-200c and miR-141 in CD18-CR cells decreased ZEB1 mRNA and protein levels that were associated with an increase in E-cadherin and a decrease in vimentin mRNA and protein levels (Supplementary Fig. S4). In contrast with the

Figure 4. Targeting MNKs decreases the protein expression of ZEB1 without reducing ZEB1 mRNA levels. A and B, CD18-CR cells were transfected with control siRNA (siCtrl) or ZEB1-specific siRNA (siZEB1) for 72 hours, and the effect on E-cadherin and vimentin protein expression was determined by Western blotting (A, left). CD18-CR cells growing on glass coverslips were transfected with siCtrl or siZEB1 for 72 hours. The cells were then stained for E-cadherin, actin using phalloidin and DAPI to counterstain the nuclei (A, right). The transfected cells were plated onto thin-layer type I collagen matrix overlaid with colloidal gold, allowed to migrate for 24 hours, and the tracks were photographed and quantified (B); **, P < 0.01. C, CD18-CR and AsPC1 cells in 3D collagen were treated with CGP57380 (2.5 μmol/L) for 72 hours and the effect on ZEB1 mRNA was determined by qRT-PCR. The effect on ZEB1 protein was determined by Western blotting. D, CD18-CR cells were transfected with control siRNA (siCtrl), MNK1 siRNA (siMNK1), MNK2 siRNA (siMNK2), or a combination of MNK1- and MNK2-specific siRNAs (siMNK1/2) for 48 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The cells were then processed for MNK1, MNK2, and ZEB1 mRNA and protein expression by Western blotting and qRT-PCR. E, CD18-CR cells on tissue culture plastic were treated with CGP57380 (5 μmol/L) for 48 hours. The cells were subjected to hypotonic lysis followed by separation of equal amounts of supernatant, as measured by OD at 260 nm, on 10% to 50% sucrose gradients and the OD at 254 nm was recorded. The OD254 is shown as a function of gradient depth. The levels of ZEB1 and GAPDH mRNA in the whole cell lysates and in the polysomal fractions were determined by qRT-PCR. The relative amounts of ZEB1 mRNA in the polysomal fractions were compared with the relative amounts of ZEB1 mRNA in the whole-cell lysates. The results are representative of three independent experiments.
Targeting the MNK effector hnRNPA1 increases ZEB1 protein without increasing ZEB1 mRNA levels

As we have found that MNK knockdown and elf4E knockdown have opposite effects on ZEB1 protein levels, it is possible that additional MNK effectors may regulate ZEB1 mRNA translation. As hnRNPA1 was previously shown to function as a translational repressor of Tnfα mRNA downstream of MNKs (15), we evaluated the extent to which the MNK effector hnRNPA1 regulated ZEB1 mRNA translation. We downregulated hnRNPA1 in CD18-CR and AsPC1 cells using siRNA and examined the effect on ZEB1 protein and mRNA levels. Significantly, downregulation of hnRNPA1 increased ZEB1 protein levels without affecting ZEB1 mRNA levels in both CD18-CR (Fig. 5D) and AsPC1 cells (Supplementary Fig. S5) that was associated with a decrease in E-cadherin and an increase in vimentin mRNA and protein levels.

The results indicate that the MNK effector hnRNPA1, similar to its regulation of Tnfα mRNA translation (15), functions as a translational repressor of ZEB1 mRNA in PDAC cells.

MNK1/2 inhibitors decrease growth of CD18-CR cells in 3D collagen and decrease Aldefluor(+) cells

We next evaluated the effect of targeting MNKs on growth of CD18-CR cells in collagen. Treatment with CGP57380 and siRNA-mediated knockdown of MNK1/2 decreased the growth of CD18-CR cells in 3D collagen (Fig. 6A and B).

As chemoresistant cells can have increased numbers of cancer stem cells (34, 35), we evaluated the presence of Aldefluor(+) cells in CD18-CR cells. Aldefluor(+) cells have previously been shown to be associated with cancer stem cells (36, 37). CD18-CR cells demonstrate increased numbers of Aldefluor(+) cells compared with CD18 cells (Fig. 6C). Significantly, treatment with the MNK inhibitor CGP57380 decreased the number of Aldefluor(+) cells in CD18-CR cells (Fig. 6D).

Treatment of human PDAC organoids with the MNK inhibitor CGP57380 increases E-cadherin mRNA levels and decreases vimentin mRNA levels without affecting ZEB1 mRNA levels

Finally, to provide in vivo support for our findings that targeting MNKs can reverse EMT, we evaluated the effect of CGP57380 on human PDAC organoids. Because these organoids can accurately model PDAC progression by recapitulating the key features of human PDAC tumors (24), we established human PDAC organoids from deidentified PDAC specimens obtained on an IRB-approved protocol using the recently published Tuveson Laboratory protocol (24). Human PDAC specimens were minced, proteolytically digested, and then embedded in Matrigel to generate human pancreatic organoids (Fig. 7A). Treatment of three different PDAC organoids with the MNK inhibitor CGP57380 did not significantly affect ZEB1 mRNA levels (Fig. 7B). Although we were not able to analyze the effect on ZEB1 protein levels due to insufficient amount of protein lysates, treatment of organoids with CGP57380 increased E-cadherin mRNA levels and decreased vimentin mRNA levels. In addition, CGP57380 inhibited growth of human PDAC organoids (Fig. 7C). These results suggest that MNK inhibitors may be able to reverse EMT in human PDAC tumors by targeting ZEB1 mRNA translation and may limit growth of PDAC tumors.

Discussion

Elevated mRNA translation, especially of genes that regulate key cellular processes, is an important feature of cancer cells (9, 10). Dysregulation of mRNA translation contributes to treatment resistance and tumor progression (17, 18). Moreover, increased elf4E phosphorylation in human PDAC tumors is associated with high-grade tumors and poor prognosis (9). Thus, there is increasing interest in understanding how enhanced mRNA translation contributes to PDAC progression. In this report, we show that the collagen microenvironment promotes MNK-dependent elf4E phosphorylation to regulate ZEB1 mRNA translation and EMT in PDAC cells.

We show that MNK inhibitors decrease migration of PDAC cells. This is in agreement with a recent report demonstrating that MNK inhibitors block migration of breast and oral cancer cells (38). Although MNK inhibitors decrease vimentin protein levels without affecting vimentin mRNA levels in breast cancer cells (38), we have found that pharmacologic and genetic targeting of MNK inhibitors decrease both vimentin protein and mRNA levels in PDAC cells. Significantly, it was recently shown that MNKs could regulate mRNA translation of EMT transcription factors. For example, treatment of mouse NMuMG breast cells with MNK inhibitors decreased TGFβ-induced Snail protein expression without affecting Snail mRNA levels (39). We have found that targeting MNKs decreases ZEB1 protein levels without affecting ZEB1 mRNA levels. Significantly, we also show treatment of human pancreatic organoids with MNK inhibitors does not affect ZEB1 mRNA levels, but increases E-cadherin mRNA levels.

Figure 5

Targeting elf4E increases ZEB1 protein and mRNA levels and decreases ZEB1-targeting miR-200c and miR-141 microRNAs. A, CD18-CR cells were transfected with control siRNA (siCtrl) or elf4E-specific siRNA (sielf4E) for 48 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The cells were processed for elf4E, ZEB1, E-cadherin, and vimentin mRNA expression by qRT-PCR and the lysates were analyzed for elf4E, ZEB1, E-cadherin, and vimentin protein expression by Western blotting. B, CD18-CR cells growing on glass coverslips were transfected with siCtrl, sielf4E, or a combination of MNK1- and MNK2-specific siRNAs (siMNK1/2) for 72 hours. The cells were then stained for actin using phalloidin and DAPI to counterstain the nuclei. C, CD18-CR cells were transfected with siCtrl or siMNK1/2 for 24 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The cells were processed for miR-200b, miR-200a, miR-200c, and miR-141 microRNAs and normalized using RNU48 as internal control. D, CD18-CR cells were transfected with control siRNA (siCtrl) or hnRNPA1-specific siRNA (sihnRNPA1) for 48 hours. The cells were then grown in 3D type I collagen (2 mg/mL) for additional 24 hours. The cells were processed for hnRNPA1, ZEB1, E-cadherin, and vimentin protein expression by Western blotting. The results are representative of three independent experiments. See also Supplementary Figs. S2–S5.
and decreases vimentin mRNA levels. Thus, our findings demonstrate that MNKS regulate EMT through modulation of ZEB1 mRNA translation.

ZEB1 plays an important role in PDAC progression. ZEB1 expression is increased in poorly differentiated human PDAC tumors and in invasive cells arising from differentiated PDAC tumors (40, 41). ZEB1 expression is increased in patients with early recurrence compared with patients with long-term remission following surgery (40). Increased ZEB1 expression is also associated with chemoresistance in pancreatic cancer cells (42, 43). Consistent with our findings that PDAC cells developing resistance to 5-FU have increased ZEB1 levels, others have shown that PDAC cells developing resistance to gemcitabine chemotherapy demonstrate increased ZEB1 expression (42, 43). These chemoresistant cells have increased numbers of cancer stem cells that can be reduced by targeting ZEB1 (40). We show that MNKS decrease the number of ALDH(+) cells in pancreatic cancer, suggesting that targeting MNKS may enable targeting of PDAC cancer stem cells.

Paradoxically, eIF4E knockdown in PDAC cells resulted in a more mesenchymal phenotype, and increased vimentin and ZEB1 mRNA and protein expression. Our findings contrast with a recent report demonstrating that targeting eIF4E in breast cancer cells inhibited TGFβ-induced vimentin and Snail expression (44). We show that the effect of eIF4E on ZEB1 levels in PDAC cells is through its effect on miR-200c and miR-141 miRNAs. We have found that eIF4E knockdown, but not MNK1/2 knockdown, decreases miR-200c and miR-141 levels in PDAC cells. Significantly, miR-200c levels are reduced in lung and lymph node metastasis compared with primary human PDAC tumors (45). In addition, reduced levels of miR-200c are associated with worse survival after pancreatic cancer surgery (46). Consistent with our findings on the effect of eIF4E knockdown on ZEB1-miR-200c in PDAC cells, a previous report had demonstrated that chronic treatment of breast cancer cells with mTORC1 inhibitor induced ZEB1 expression through repression of miR-200c (47). As mTOR inhibitors block eIF4E function by preventing phosphorylation-dependent release of 4E-BP (eIF4E binding protein) from eIF4E.
(48, 49), it is possible that mTOR regulates ZEB1 through an eIF4E-dependent mechanism.

Although eIF4E knockdown decreased the levels of miR-200c and miR-141 miRNAs, eIF4E knockdown did not affect the levels of miR-200b and miR-200a miRNAs, suggesting that the effect of eIF4E knockdown is specific for miR-200c and miR-141 miRNAs. It is known that miR-200b and miR-200a are part of a cluster on chromosome 1, whereas miR-200c and
miR-141 belong to a cluster on chromosome 12 (32, 33). Significantly, it has been shown that these two clusters can be differentially regulated, resulting in differential expression of miR-200b-200a and miR-200c-141 primary miRNA (pri-miRNA) transcripts. For example, in HMLE breast cells, polycomb group proteins EZH2 and SUZ12 associate with and silence miR-200b-200a-429 cluster, but do not silence the miR-200c-141 cluster (33). However, we have found that eIF4E knockdown in pancreatic cancer cells did not affect the levels of pri-miRNA transcripts of miR-200b, miR-200a, or miR-200c-141, suggesting that the differential effects of eIF4E knockdown on mature miR-200 miRNAs is not at the level of transcription of these miRNAs. Moreover, eIF4E knockdown also did not affect the levels of miRNA-processing proteins, Drosha and Dicer (Supplementary Fig. S3). Future experiments will determine how eIF4E knockdown preferentially decreases the levels of miR-200c-141 miRNAs in pancreatic cancer cells.

Although eIF4E is the best-characterized phosphorylation target of MNKs, an additional MNK effector is hnRNPA1, a nuclear protein that is involved in regulation of alternative splicing, mRNA export, and mRNA translation (50, 51). Previously, it was shown that MNK-mediated phosphorylation of hnRNPA1 decreases binding of hnRNPA1 to TNFa mRNA and thereby increases translation of TNFa mRNA (15), demonstrating that hnRNPA1 functions as a translational repressor of TNFa mRNA. The hnRNPA1 protein has affinity for AU-rich sequences, in particular the AUUUA sequence, which is contained within the 3′-untranslated region (3′-UTR) of eukaryotic genes (52).

Importantly, ZEB1 has 9 AUUUA sequences in its 3′-UTR (AREsite). Significantly, we have found that knockdown of hnRNPA1 using siRNA increases ZEB1 protein levels without affecting ZEB1 mRNA levels, indicating that the MNK effector hnRNPA1 also functions as a translational repressor of ZEB1 mRNA in PDAC cells.

Overall, we demonstrate that MNKs play an important role in PDAC progression. Targeting MNKs can reverse EMT, decrease migration, limit growth of PDAC cells, and may reduce pancreatic cancer stem cells. We also demonstrate that targeting MNKs, in contrast with targeting eIF4E, does not affect ZEB1-targeting miRNAs (Fig. 7D, model). Thus, MNKs may be particular appropriate targets to consider in the treatment of this deadly cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: F.D. Eckerdt, L.C. Platanias, H.G. Munshi Development of methodology: K. Kumar, H.G. Munshi Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Kumar, C.R. Chow, A.D. Arslan, B. Kwok, D.J. Bentrem, H.G. Munshi Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Kumar, C.R. Chow, A.D. Arslan, B. Kwok, F.D. Eckerdt, L.C. Platanias, H.G. Munshi Writing, review, and/or revision of the manuscript: K. Kumar, C.R. Chow, F.D. Eckerdt, L.C. Platanias, H.G. Munshi Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Kumar, K. Ebine, D.J. Bentrem, H.G. Munshi Study supervision: H.G. Munshi

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